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13. ABSTRACT *(Maximum 200 words)*

Three areas of research on the Type II DHFR have been pursued. These were (i) analysis of structure and mechanism of the enzyme; (ii) stability effects on the protein and variants *in vivo* and *in vitro*; and (iii) plasmid constructs of mutants and expression of mutants. Studies of the first have shown that the stereochemistry of hydride transfer from NADPH to dihydrofolate placed this enzyme as one of the A-stereospecific class of dehydrogenases. The interaction of the enzyme with cofactors has been studied by equilibrium dialysis, proton NMR spectroscopy and differential scanning calorimetry. Two types of complexes have been found, and a shift of conformation between 5.0 and 5.9 was observed. In the mutant work, several mutants in the proposed active site have been formed. Some were inactive *in vivo*. In certain cases (e.g., Glu 58 → Gln 58) the enzyme was rather unstable to protease attack *in vivo*. This factor was even more pronounced in *sup*$^+$ strains.

14. SUBJECT TERMS

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GENETIC ENGINEERING OF A TYPE II DHFR

FINAL REPORT

G.N. BENNETT

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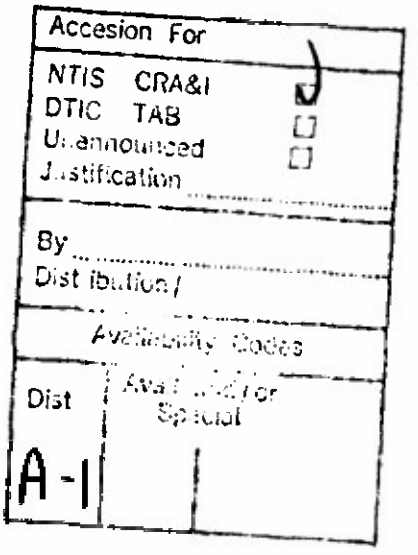
3. List of Appendices

   1) Copy of Brito, R.M.M., Reddick, R., Bennett, G.N., Rudolph, F.B., and Rosevear, P.F., Characterization and Stereochemistry of Cofactor Oxidation by a Type II Dihydrofolate Reductase, *Biochemistry*, **29**, 9825-9831 (1990).

   2) Copy of Vermersch, P.S., and Bennett, G.N., Stability of mutant Type II dihydrofolate reductase proteins in suppressor strains, *J. Biotechnology*, **19**, 49-66 (1991).



4. Body of Report

A. Type II dihydrofolate reductases (DHFRs) encoded by the R67 and R388 plasmids are quite different in sequence from known chromosomal DHFRs. These plasmid derived DHFRs are responsible for conferring trimethoprim resistance to the host strain. One of the features that makes the Type II DHFR an attractive system for enzymological study of the catalytic properties of the protein is the unique and primitive character of this enzyme, as illustrated by the following points. The Type II DHFR has a low turnover rate compared to other DHFRs. It can use either α or β NADPH, revealing a less stringent substrate specificity than other reductases. It is a very small protein, especially when the sizes of its two substrates are considered, so it represents a minimal enzyme that should allow engineered improvements based on available information of the structures and mechanisms of other DHFRs. Thus not only can changes be made in the amino acid sequence which eliminate or reduce the activity or specificity of the enzyme (as has been the case with most other reported studies),.but, more importantly, changes can be sought which enhance the specificity, the binding of inhibitors, or the catalytic rate of the enzyme. This protein thus provides a model system for long term studies related to designing a better active site.

Another feature, separate from the catalytic aspect, is the physical structure of the protein, particularly the interactions that confer its unusual stability to heat and denaturants. Since this protein is a simple β-barrel with all A $\rightarrow$ B loops it serves as a natural construct for studies of this basic protein structure, and of the interactions which stabilize it. The basic barrel structure can be considered as stable scaffold onto which functional loops and specially located side chains can be placed. The tendency of the protein to associate is also of interact and enables it to be considered as a model for research into the interactions involved in protein:protein contacts.

B. Summary of Results

Three areas of research on the Type II HFR have been pursued. These are: (i) analysis of structure and mechanism of the enzyme; (ii) stability effects; and (iii) oligonucleotide

and plasmid construction for expression of mutants.

In the area of protein stability, we have used extracts of a number of isogenic *sup*^{0} or *sup* strains to investigate the degradation of a Glu 58 → Gln 58 mutant DHFR. These strains have little effect on the natural enzyme and the DHFR is stable in these strains. After preparation of log phase cultures of the various strains, extracts were made by sonication and the protein content analyzed. The extracts were incubated with a constant amount of purified mutant (or natural) DHFR and the products separated by SDS-polyacrylamide gel electrophoresis. After staining with Coomassie clue and destaining, the gels were dried in frames between clear film sheets. The gel bands are scanned and the relative amount of undegraded DHFR was obtained *vs* time with each extract. The differences in degradation between the strains were plotted. Also investigated was the role of ATP in the process, since certain proteases require ATP for digestion (e.g., lon), we have determined that the addition of ATP or its removal by filtration of the extract does not affect the degradation of the mutant DHFR.

Investigations with several common laboratory *E. coli* strains including *htpR* and *lon* strains bearing plasmids expressing the Gln 58 DHFR indicated a correlation of rapid degradation with the presence of a *sup*$^+$ phenotype in the strain rather than with a *lon*$^+$ phenotype. The *sup*^{0} strain MC1061(p3) was transformed with a series of plasmids containing the Gln 58 DHFR gene with and without an additional *sup*F gene, and expression levels were compared. The *sup*F$^+$ constructs exhibited little accumulation of the Gln 58 DHFR, while reasonable levels were found in the *sup*^{0} cases. Experiments with extracts of plasmid-free *sup*$^+$ and *sup*^{0} strains showed rapid degradation by certain strains compared to MC1061(p3).

These studies so far suggest that certain mutant proteins are more readily degraded in *sup*$^+$ strains. This would be important in the consideration of the proper strain to use to produce an engineered protein. This also has widespread implications as many laboratory strains routinely used as hosts in a recombinant protein production are *sup*$^+$ and these may

not be the best hosts for this use.

In another route to increasing the stability of labile DHFR derivatives, mutagenesis of a strain bearing a N-terminally shortened Gln 58 DHFR was preformed. Selection and analysis of a trimethoprim resistant stable mutant showed that this DHFR gene contained a triple repeat of leu-pro-ser in the enzymatically non-essential N-terminal portion of the protein. This suggests N-terminal additions or longer versions of the proteins may help stabilize it *in vivo*.

A derivative of R388 DHFR, RBG200, which has six additional amino acids, Thr-Thr-Ser-Arg-Thr-Leu at the carboxy terminus in addition to the 78 amino acids of the R388 DHFR, has been cloned and overproduced (Vermersch *et al.*, 1986). A rapid purification procedure has been developed which yields milligram quantities of homogeneous RBG200 DHFR exhibiting a specific activity 1.5 fold greater than that previously reported for the purified R388 protein (Amyes and Smith, 1976). The pH *vs* activity profile and the native molecular weight of RBG200 DHFR were found to be similar to that previously reported for other Type II DHFRs, and significantly different from the known chromosomal DHFRs. Stereospecifically labeled [4S-^{2}H, 4R-^{1}H]NADPH was synthesized and used to determine the stereospecificity of NADPH oxidation by RBG200 DHFR. RBG200 DHFR was found to specifically transfer the pro-R hydrogen of NADPH to dihydrofolate, making it a member of the A-stereospecific class of dehydrogenases (Pastore and Friedkin, 1962). Thus, although RBG200 DHFR is sequence and structurally different from the host enzyme, both enzymes catalyze identical hydrogen transfer reactions. Equilibrium dialysis and proton NMR spectroscopy were used to further study the enzymes. RBG200 DHFR was found to bind approximately one NADP^{+} per tetramer, suggesting that the enzymatically active form of the enzyme is a tetramer. Two distinct binary RBG200 DHFR-NADP^{+} complexes were detected by monitoring the ^{1}H NMR chemical shifts and line widths of the coenzyme in the presence of RBG200 DHFR (Brito *et al.*, 1991).

Conformation I slowly interconverts to a second more stable binary complex,

Conformation II. The binding of $NADP^+$ to RBG200 DHFR in the second binary complex was relatively weak, $K_D = 1.9 \pm 0.4$ mM. Transferred NOEs were used to determine the conformation of $NADP^+$ bound to RBG200 DHFR. The initial slope of the NOE build-up curves, measured from the intensity of the cross-peaks as a function of the mixing time in NOESY spectra allowed interproton distances on enzyme-bound $NADP^+$ to be estimated. The experimentally measured distances were used to define upper and lower bound distance constraints between proton pairs in distance geometry calculations. All $NADP^+$ structures consistent with the experimental distance bounds were found to have a *syn* conformation about the nicotinamide-ribose ($\chi = 94 \pm 26°$) and an *anti* conformation about the adenine-ribose ($\chi = -92 \pm 21°$) glycosidic bonds. From the known stereochemistry of hydride transfer and the conformation of the enzyme-bound cofactor, a model was proposed for the orientation of cofactor and substrate at the active site of RBG200 DHFR. The conformation of $NADP^+$ bound to RBG200 DHFR in the initial binary complex was qualitatively evaluated at 5° C, in order to decrease the rate of conformational interconversion to the second complex. The ratio of cross-peak intensities as well as the pattern of observed NOEs were only consistent with *syn* and *anti* conformations about the nicotinamide-ribose and adenine-ribose bonds, respectively, in this initial complex. However, conformational differences between enzyme-bound $NADP^+$ in the two binary complexes could be detected.

Further studies on the DHFR using specific incorporation of C^{13}-labeled amino acids require the induction of the protein in appropriate auxotrophs. The use of pRBG200 as the plasmid for labeling experiments leads to mixed labeling due to the slow accumulation of the protein as it enters stationary phase. Efforts to increase quantity and labeling extent are ongoing. Effects to prepare an inducible construct of the RBG200 DHFR are also underway.

The study of the protein and complexes by differential scanning colorimetry has begun in collaboration with J. Sturtevant J.E. Cadbury, Yale University using a large preparation of the RBG200 DHFR that we sent them. Isothermal microcalorimetric titration of β-NADPH with the protein was used to study binding of the cofactor under conditions where

the NADPH should be its most stable. Findings include a value of 1 NADPH binding site per tetramer in agreement with the NMR data. However, the determinations of the concentrations of the DHFR have more error than the NADPH term. Also, there may be some equilibrium between various oligomeric forms of the enzyme. The binding of NMNH seems to be different than NADPH and may indicate some lack of specificity. This would fit with the unusual ability of the enzyme to use the α or β form of the cofactor. Other further studies are planned to correlate this type of analysis with the results obtained from NMR and other experiments.

C. Brito, R.M.M., Reddick, R., Bennett, G.N., Rudolph, F.B., and Rosevear, P.R., Characterization and stereochemistry of cofactor oxidation by a type II dihydrofolate reductase, *Biochemistry*, **29**, 9825-9831 (1990).

Vermersch, P.S., and Bennett, G.N., Stability of mutant Type II dihydrofolate reductase proteins in suppressor strains, *J. Biotechnology*, **19**, 49-66 (1991).

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Vermersch, P.S., Klass, M.R., and Bennett, G.N., (1986) *Gene*, **41**, 289-297.

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Pastore, E.J. and Friedkin, M. (1962) *J. Biol. Chem.* **237**, 3802.

Brito, R.M.M., Rudolph, F.B., and Rosevear, P.F., (1991) *Biochemistry*, **30**, 1461-1469.

7. Appendix material

Reprinted from Biochemistry, 1990, 29.

Characterization and Stereochemistry of Cofactor Oxidation by a Type II Dihydrofolate Reductase†

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ABSTRACT: Type II dihydrofolate reductases (DHFRs) encoded by the R67 and R388 plasmids are different both in sequence and in structure from known chromosomal DHFRs. These plasmid-derived DHFRs are responsible for conferring trimethoprim resistance to the host strain. A derivative of R388 DHFR, RBG200, has been cloned and overproduced [Vermersch, P. S., Klass, M. R., & Bennett, G. N. (1986) *Gene 41*, 289]. With this cloned and overproduced protein, a rapid purification procedure has been developed that yields milligram quantities of apparently homogeneous RBG200 DHFR with a specific activity 1.5-fold greater than that previously reported for the purified R388 protein [Amyes, S. G. B., & Smith, J. T. (1976) *Eur. J. Biochem. 61*, 597]. The pH versus activity profile and the native molecular weight of RBG200 DHFR were found to be similar to those previously reported for other type II DHFRs but different from those of the known chromosomal DHFRs. Stereospecifically labeled [4(*S*)-^{2}H,4(*R*)-^{1}H]NADPH was synthesized and used to determine the stereospecificity of NADPH oxidation by RBG200 DHFR. RBG200 DHFR was found to specifically transfer the *pro-R* hydrogen of NADPH to dihydrofolate, making it a member of the A-stereospecific class of dehydrogenases. Thus, although RBG200 DHFR is different both in sequence and in structure from known chromosomal enzymes, both enzymes catalyze identical hydrogen-transfer reactions. Two distinct binary RBG200 DHFR–$NADP^+$ complexes were detected by monitoring the ^{1}H NMR chemical shifts and line widths of the coenzyme in the presence of RBG200 DHFR. Addition of $NADP^+$ to the enzyme results in the formation of an initial binary complex (conformation I) which interconverts to a more stable binary complex (conformation II). At 25 °C the apparent first-order rate constant for the interconversion between conformations I and II was determined to be approximately 1.0×10^{-4} s^{-1}. Conformations I and II are characterized by upfield and downfield chemical shifts of the nicotinamide proton resonances from their positions in the free coenzyme, respectively. Changes in the ^{1}H NMR chemical shifts of the upfield-shifted methyl resonances of RBG200 DHFR were also observed upon $NADP^+$ binding and accompanying the interconversion between conformations I and II. The relevance of these two distinct binary conformations in coenzyme binding and catalysis remains to be determined.

Dihydrofolate reductase (DHFR;[1] EC 1.5.1.3) catalyzes a central reaction in one-carbon metabolism, the NADPH-dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. Chromosomal DHFRs are the target of a number of antifolate agents which function by inhibiting the enzyme and thus depleting the metabolic pool of one-carbon units necessary for normal cellular function. The discovery and use of the antifolate agents trimethoprim (TMP) and methotrexate (MTX) in clinical cases as antibacterial and antitumor agents led to the identification of different types of DHFRs, types I–IV, less sensitive to these antifolate compounds (Fleming et al., 1972; Amyes & Smith, 1974; Skold & Widh, 1974).

The type II enzymes were found to be specified by plasmids R67 and R388, which confer TMP resistance (Amyes & Smith, 1974; Pattishall et al., 1977). Isolation of type II enzymes and analysis of their enzymological properties showed them to be very insensitive to TMP and MTX, although they have K_m values for NADPH and dihydrofolate similar to those of the chromosomal enzymes (Pattishall et al., 1977). A comparison of the amino acid sequences of the R67- and R388-specified DHFRs showed homology over their 78 amino acid length. Of the 17 changes that occurred, 11 were from the N-terminus to amino acid 23, and two others were at the extreme C-terminus (Zolg et al., 1978; Zolg & Hanggi, 1981). This is in contrast to all known DHFRs from bacterial and mammalian sources which have been shown to be monomers of approximately 18 kDa (Kraut & Matthews, 1986).

The crystallographic structure of a dimeric form of R67 DHFR has been solved to 2.8-Å resolution (Matthews et al., 1986), although the active form of the enzyme appears to be a tetramer (Zolg & Hanggi, 1981; Smith et al., 1979). Crystallographic analysis of the dimer showed that each 78-residue subunit is folded into a 6-stranded antiparallel β-barrel. The β-strands from two subunits form a third β-barrel at the dimer interface. Matthews et al. (1986) have proposed that NADPH binds along a lengthwise cleft between the subunits. This structural motif suggests that the type II DHFRs have a pyridine nucleotide binding site different from those of chromosomal DHFRs and other known pyridine nucleotide binding proteins.

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[1] Abbreviations: DHFR, dihydrofolate reductase; TMP, trimethoprim; MTX, methotrexate; DHF, 7,8-dihydrofolate; EDTA, ethylenediaminetetraacetic acid; DTT, 1,4-dithiothreitol; DSS, sodium 2,2-dimethyl-2-silapentane-5-sulfonate; SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; PMSF, phenylmethanesulfonyl fluoride.

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A derivative of R388 DHFR, RBG200, having the sequence Thr-Thr-Ser-Arg-Thr-Leu- at the carboxy terminus in addition to the 78 amino acids of the R388 DHFR has been cloned and overproduced (Vermersch et al., 1986). A nondenaturing procedure for the rapid purification of milligram quantities of this derivative has been developed. RBG200 DHFR has been shown to have physical and chemical properties similar to those of the native R388 DHFR, making it an ideal system for biochemical studies aimed at elucidating the proposed novel mode of protein–cofactor interaction and the molecular basis for resistance to the common antifolate agents. With this purification procedure, RBG200 DHFR is soluble to at least 2 mM, making structural studies feasible. We have initiated biochemical studies required for a detailed understanding of the protein–cofactor interaction. The stereochemistry of hydride transfer from NADPH to DHF has been shown to be the same as that observed for chromosomal DHFRs. In addition, the existence of two distinct binary RBG200 DHFR–$NADP^+$ complexes has been observed by ^1H NMR spectroscopy.

Materials and Methods

Materials. Isocitrate dehydrogenase and $NADP^+$ were purchased from Sigma. ^2H_2O was purchased from Cambridge Isotope Laboratories. DEAE-Sepharose Fast Flow was purchased from Pharmacia. All other chemicals were of highest quality commericially available.

Enzyme Assay. Dihydrofolate reductase activity was assayed as described by Smith and Burchall (1983), except the buffer used was 50 mM potassium phosphate, 1 mM EDTA, and 1 mM DTT at pH 5.9. One enzyme unit is the quantity of enzyme required to convert 1 μmol of NADPH and dihydrofolate to $NADP^+$ and tetrahydrofolate per minute calculated on the basis of an absorption coefficient of 12 300 L mol^{-1} cm^{-1} at 340 nm (Smith & Burchall, 1983). Protein concentration was estimated by the method of Bradford (1976).

Bacterial Strains and Growth. Escherichia coli C600 cells bearing the plasmid RBG200 (Vermersch et al., 1986) were grown in minimal media in the presence of 0.2% casein amino acids and 15 mg/L trimethoprim. Cells were harvested by centrifugation, mixed with an equal weight of 50 mM Tris buffer containing 10% sucrose at pH 7.5, poured into liquid nitrogen, and stored at −70 °C.

Buffers. Cell lysis buffer contained 50 mM Tris, pH 8.0, 5% glycerol, 2 mM EDTA, 1 mM DTT, 0.24 M NaCl, 1.4 mM 2-mercaptoethanol, and 0.1 mM PMSF. TGED buffer is 10 mM Tris, pH 8.0, 5% glycerol, 0.1 mM EDTA, 0.2 M NaCl, 0.1 mM PMSF, and 1 mM DTT. TME buffer contained 10 mM Tris, pH 8.5, 10 mM 2-mercaptoethanol, and 1 mM EDTA. PMSF was added to the buffers immediately prior to use.

Purification of RBG200 DHFR. The following procedure is for 20 g of *E. coli* C600 cells bearing the plasmid pRBG200 DHFR. The basic steps consist of cell lysis, streptomycin sulfate precipitation, ammonium sulfate precipitation, and DEAE-Sepharose chromatography. All steps are carried out at 4 °C unless noted otherwise.

(*I*) *Cell Lysis.* Frozen cells, 20 g of frozen cell paste (1:1), are stirred at room temperature in 55 mL of lysis buffer until homogeneous. At 4 °C, 20 mg of lysozyme is added and the suspension stirred for 25 min. Freshly made 4% sodium deoxycholate is added to a final concentration of 0.1%; the solution is stirred an additional 2 min and incubated for an additional 20 min. To this solution, 55 mL of TGED buffer and 100 mL of 50 mM Tris-HCl, pH 7.5, containing 10 mM 2-mercaptoethanol are added with stirring, and the chromosomal DNA is sheared in a blender at medium speed for 30 s. The cell lysate is then centrifuged at 10000*g* for 45 min. The supernatant is fraction I.

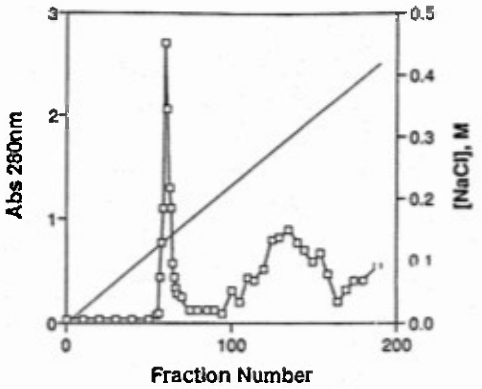

FIGURE 1: Ion exchange chromatography of RBG200 DHFR on DEAE-Sepharose. The dialyzed 35–50% ammonium sulfate fraction was applied to a 2.5 × 45 cm DEAE-Sepharose Fast Flow column equilibrated in TME buffer and washed in TME buffer. RBG200 DHFR was eluted with a 1.6-L gradient from 0 to 0.5 M NaCl in TME buffer. A flow rate of 40 mL/h was maintained throughout, and 8-mL fractions were collected. The RBG200 DHFR elutes at approximately 0.15 M NaCl with a specific activity of 2.8 units/mg.

(*II*) *Streptomycin Sulfate Precipitation.* Streptomycin sulfate is added dropwise to fraction I with constant stirring to a final concentration 1%. After complete addition of the streptomycin sulfate, the suspension is stirred for an additional 15 min and then centrifuged at 10000*g* for 15 min. The nucleic acid precipitate is discarded, and the supernatant, fraction II, is considered the crude extract.

(*III*) *Ammonium Sulfate Precipitation.* Solid ammonium sulfate is slowly added to fraction II with constant stirring over a 30-min period to 35% saturation. The suspension is stirred for 30 min and centrifuged at 10000*g* for 45 min. The precipitate is discarded, and solid ammonium sulfate is slowly added over a 30-min period to 50% saturation. After complete addition of the ammonium sulfate, the suspension is stirred for an additional 30 min. The ammonium sulfate precipitate is collected by centrifugation at 10000*g* for 45 min. The supernatant is discarded. The precipitate is dissolved in 20 mL of TME buffer and dialyzed twice against 3 L of TME buffer to give fraction III.

(*IV*) *DEAE-Sepharose Chromatography.* Fraction III is centrifuged at 10000*g* for 15 min and the supernatant loaded onto a 2.5 × 45 cm DEAE-Sepharose Fast Flow column equilibrated in TME buffer. After the supernatant has been loaded onto the column, the column is washed with 1 L of TME buffer. RBG200 DHFR is eluted with a 1.6-L linear gradient from 0 to 0.5 M NaCl in TME buffer. Dihydrofolate reductase elutes at approximately 0.15 M NaCl (Figure 1, fractions 55–66) with a specific activity of 2.8 units/mg (Table I). The purity of the enzyme was constant across the peak, as judged by SDS–polyacrylamide gel electrophoresis. An SDS–polyacrylamide gel of the pooled fraction from the DEAE-Sepharose column revealed one major band (>95%) corresponding to the known subunit molecular weight of RBG200 DHFR (Vermerscb et al., 1986; Figure 2). The purified RBG200 DHFR was dialyzed against 5 mM potassium phosphate, pH 5.9, lyophilized, and stored at −20 °C with no measurable loss of activity over 3 months.

Determination of the Stereochemistry of NADPH Oxidation. [4-^2H]$NADP^+$ was prepared by alkaline cyanide treatment of $NADP^+$ in ^2H_2O according to the procedure of San Pietro (1955). ^1H NMR spectroscopy was used to confirm

Table 1: Purification of RBG200 DHFR

	total protein (mg)	total act. (units)	sp act. (units/mg)	x-fold purification	% recovery
crude lysate	671	217	0.3		100
ammonium sulfate precipitation	378	206	0.5	1.7	95
DEAE-Sepharose	31	86	2.8	9.3	40

the position of the isotopic label and estimate the extent of deuteration (>95%). [4(*S*)-^{2}H,4(*R*)-^{1}H]NADPH was prepared with isocitrate dehydrogenase. The reaction mixture contained 50 mM Tris-HCl buffer at pH 7.2, 10 mM isocitric acid, 5 mM [4-^{2}H]NADP$^+$, 50 μM $MnSO_4$, and 2 units of isocitrate dehydrogenase in a final volume of 1.5 mL. The mixture was incubated for 2 h at 37 °C and the extent of reaction monitored by the increase in absorption at 340 nm. The reaction mixture was diluted to 50 mL with 10 mM triethylammonium bicarbonate buffer, pH 7.5, and loaded onto a 1.5 × 90 cm Pharmacia Fast Flow DEAE-Sepharose column equilibrated in 10 mM triethylammonium bicarbonate buffer, pH 7.5. The labeled NADPH was eluted with a 500-mL linear gradient from 10 to 500 mM triethylammonium bicarbonate, pH 7.5, at a flow rate of 1.5 mL/min. Fractions having A_{260}/A_{340} ratios of approximately 2.5 were pooled, and the isotopically labeled NADPH precipitated with a 10-fold excess of cold acetone. The chemical shift and coupling constant of the C4 methylene proton of the isotopically labeled NADPH confirmed the stereochemistry at C4 to be 4(*S*)-^{2}H,4(*R*)-^{1}H. The [4(*S*)-^{2}H,4(*R*)-^{1}H]NADPH (70 μM) was incubated with RBG200 DHFR (0.11 mg) in a reaction mixture which contained 45 mM potassium phosphate buffer, pH 5.9, 120 μM dihydrofolate, and 12 mM 2-mercaptoethanol for 1 h at 22 °C. The reaction mixture was diluted with 50 mL of 10 mM triethylammonium bicarbonate buffer, pH 7.5, and loaded onto a 1.5 × 30 cm Pharmacia Fast Flow DEAE-Sepharose column. NADP$^+$ was eluted with a 500-mL linear gradient from 10 to 500 mM triethylammonium bicarbonate, pH 7.5. Fractions containing the purified NADP$^+$ were pooled, and the NADP$^+$ was collected by precipitation with a 10-fold excess of cold acetone. The precipitated NADP$^+$ was dissolved in ^2H_2O for analysis by ^{1}H NMR. The stereochemistry of oxidation of [4(*S*)-^{2}H,4(*R*)-^{1}H]NADPH by RBG200 DHFR was not monitored continuously in the NMR spectrometer due to interference by dihydrofolate, tetrahydrofolate, and aromatic amino acid residues of the protein. Proton NMR spectra were obtained at 22 °C on a GN500 NMR spectrometer. Spectra were obtained by collecting 128 transients with 16K data points, a spectral width of 5000 Hz, a 90° pulse, and a 5-s repetition rate. DSS was used as an internal standard and the pH of the samples adjusted to 7.0.

Enzyme Preparation for NMR Studies. Purified RBG200 DHFR, from a lyophilized powder, was dissolved in 50 mM potassium phosphate buffer, pH 6.0, in ^2H_2O. The solution was lyophilized and redissolved in ^2H_2O to remove most of the residual HDO and to deuterate exchangeable protons. All buffer and reagent solutions used in the NMR studies were treated with Chelex 100 before use to remove trace metal contaminants. RBG200 DHFR was found to retain at least 90% of its original activity at the end of the NMR experiments.

Results and Discussion

Purification of RBG200 DHFR. The purification procedure for RBG200 DHFR from *E. coli* C600 cells carrying the plasmid pRBG200 is summarized in Table 1, and the SDS-polyacrylamide gel electrophoresis monitoring the purification from crude extracts is shown in Figure 2. The overall purification from 10 g of cell paste (20 g of 1:1 mixture of cells and Tris/sucrose buffer) is 31 mg of apparently homogeneous enzyme, representing a 40% yield. The specific activity of RBG200 DHFR, 2.8 units/mg, is significantly higher than the specific activity previously reported for the similar R388 enzyme, 1.5 units/mg (Zolg & Hanggi, 1981). The increased specific activity of RBG200 DHFR, a derivative of the R388 enzyme, most likely results from the more gentle and rapid purification procedure used to purify the overproduced protein. The previous purification procedure included a step where the enzyme is denatured by boiling in 6 M guanidinium chloride and passed over a gel filtration column in 6 M urea (Zolg & Hanggi, 1981). RBG200 DHFR purified according to the procedure of Zolg and Hanggi (1981) was also found to have a lower specific activity, approximately 1.5 units/mg. Thus, it is possible that once the type II DHFR is partially unfolded it does not refold completely to its native conformation. Preliminary circular dichroism studies of RBG200 DHFR at pH 5.9 show a significant amount of α-helical structure, in contrast to the antiparallel β-sheet structure obtained by crystallography for the dimeric form of R67 DHFR (Matthews et al., 1986).

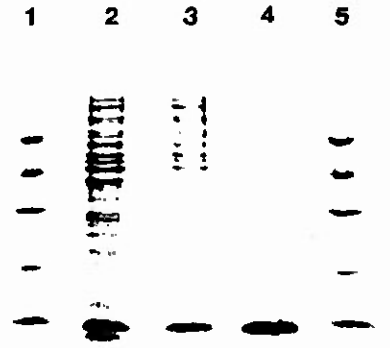

FIGURE 2: The 20% SDS–polyacrylamide gel electrophoresis monitoring the purification of RBG200 DHFR from *E. coli* C600 cells carrying the plasmid RBG200. (Lanes 1 and 5) Molecular mass standards: bovine serum albumin (68 kDa), hen egg albumin (45 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa). (Lane 2) Supernatant from streptomycin sulfate precipitation. (Lane 3) Dialyzed fraction from the 35–50% ammonium sulfate cut. (Lane 4) RBG200 DHFR eluted from the DEAE-Sepharose column. The gel was stained with Coomassie Brilliant Blue.

With the overproduction of RBG200 DHFR and the simplified purification scheme, large amounts of RBG200 DHFR can be prepared for biochemical and NMR studies aimed at understanding the unique structural properties of this protein. In addition, this type of purification scheme is necessary before efficient incorporation of stable isotopes into RBG200 DHFR becomes practical for detailed magnetic resonance studies. A major advantage of RBG200 DHFR is its greatly increased solubility (>2 mM) compared to that of the R67 or R388 proteins.

pH Profile of RBG200 DHFR. The pH versus activity profile of RBG200 DHFR is shown in Figure 3. RBG200 DHFR has a sharp pH optimum at 5.9, similar to that observed for the R388 enzyme (Amyes & Smith, 1976). At pH 7.5 the enzyme exhibits approximately 25% of its maximal activity. However, at pH 5.0, less than 1 pH unit from the pH of maximal activity, RBG200 DHFR exhibits only 5% of

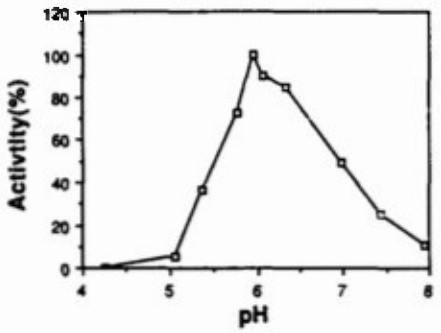

FIGURE 3: pH versus activity profile for RBG200 DHFR. The percentage of RBG200 DHFR activity is shown at different pH values. Potassium phosphate buffer, 50 mM, containing 1 mM EDTA and 1 mM DTT was used for the pH range 4.2–7.9. The pH was measured prior to, and immediately after, monitoring of the absorbance change of reaction with a microelectrode and found to vary less than ±0.05 unit. Each data point is the average of three separate assays.

its maximal activity. The sharp decrease in activity over approximately 0.9 pH unit may result from protonation of a carboxylate group essential in catalysis or in maintaining the proper quaternary or tertiary structure of the enzyme. This is in contrast with the *E. coli* chromosomal DHFR which exhibits a broad pH optimum between pH 5.5 and pH 7.5 (Matthews & Sutherland, 1965; Amyes & Smith, 1976).

Native Molecular Mass of RBG200 DHFR. The native molecular mass of the purified enzyme was determined at both pH 5.9 and pH 5.0 by HPLC gel filtration on a Superose 12 column (Figure 4). RBG200 DHFR yielded a single peak with an apparent molecular mass of 32 and 35 kDa at pH 5.9 and 5.0, respectively (Figure 4). From the subunit molecular mass, 8.8 kDa (Vermersch et al., 1986), the native enzyme exists in solution as a tetramer at both pH values. This is consistent with the subunit composition previously found for the R67 enzyme (Smith et al., 1979). Thus, it appears that type II plasmid derived DHFRs are tetramers composed of four identical subunits, in contrast with all known chromosomal DHFRs which exist as monomers with molecular masses of approximately 18 kDa (Smith et al., 1979). Gel filtration experiments at pH 5.0 (Figure 4B) also demonstrate that RBG200 DHFR maintains its quaternary structure at low pH and rapid loss of enzymatic activity between pH 5.9 and pH 5.0 (Figure 3) is not the result of dissociation of the active enzyme into inactive monomers or dimers.

Hydride-Transfer Stereospecificity of RBG200 DHFR. [4(*S*)-^{2}H,4(*R*)-^{1}H]NADPH was synthesized from [4-^{2}H]-NADP$^+$ with isocitrate dehydrogenase and isocitric acid. It is well established that isocitrate dehydrogenase transfers a hydrogen from the C2 of isocitric acid to the A face (*pro-R* position) of NADPH (Englard & Colowick, 1957; Nakamoto & Vennesland, 1960; Freudenthal et al., 1973). Additionally, the stereochemistry and isotopic purity of the synthesized NADPH were confirmed by ^{1}H NMR spectroscopy (Arnold et al., 1976; Seyama et al., 1977; Arnold & You, 1978).

The synthesized [4(*S*)-^{2}H,4(*R*)-^{1}H]NADPH was incubated with dihydrofolate and RGB200 DHFR, and the enzymatically oxidized NADPH was isolated by column chromatography. This was necessary to prevent interference in the ^{1}H NMR spectrum of NADP$^+$ from the protein, NADPH, dihydrofolate, and tetrahydrofolate. If the type II plasmid DHFR transferred the A-side (*pro-R*) hydrogen, the isolated NADP$^+$ would contain deuterium at the N4 position. However, if the enzyme transferred the B-side (*pro-S*) hydrogen, the isolated NADP$^+$ would contain a proton at the N4 position. The ^{1}H NMR spectra of the aromatic region of commercial NADP$^+$ and NADP$^+$ isolated from incubation of [4(*S*)-^{2}H,4(*R*)-^{1}H]-

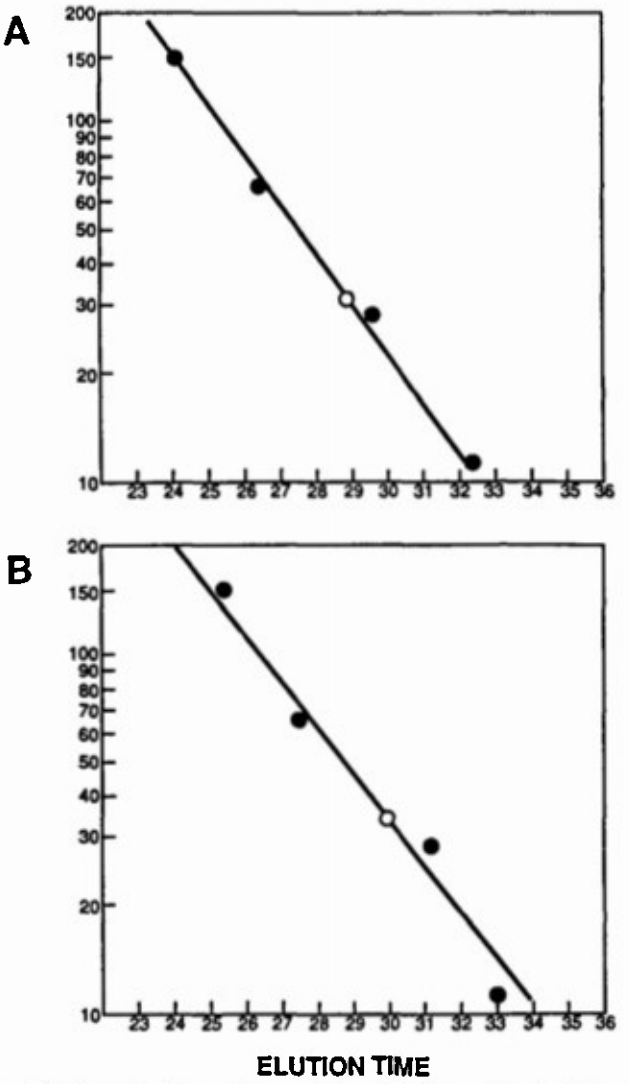

FIGURE 4: Estimation of the native molecular mass of purified RBG200 DHFR at pH 5.9 (A) and pH 5.0 (B). Gel filtration was performed on a Superose 12 (Pharmacia) HPLC column, 10 × 300 mm, at 25 °C in 50 mM potassium phosphate buffer, pH 5.9, containing 50 mM NaCl (A) or in 50 mM potassium phosphate buffer, pH 5.0, containing a 50 mM NaCl (B). A flow rate of 0.5 mL/min was used at both pH values. A calibration curve was prepared with the following molecular mass standards (●): alcohol dehydrogenase (150 kDa), bovine serum albumin (68 kDa), carbonic anhydrate (29 kDa), and cytochrome *c* (12.4 kDa). Native RBG200 DHRF (○) eluted at a position corresponding to a molecular mass of 32 (A) and 35 kDa (B), respectively.

NADPH with RBG200 DHFR are compared in Figure 5. The isolated NADP$^+$ is shown to contain deuterium at N4 (Figure 5A). Therefore, RBG200 DHFR specifically removes the *pro-R* hydrogen, leaving behind the *pro-S* deuterium. Chromosomal DHFRs are known to transfer the *pro-R* hydrogen from NADPH to dihydrofolate (Charlton et al., 1979). Thus, the stereochemistry of hydrogen transfer from NADPH to dihydrofolate by two evolutionary distinct reductases is identical. This was predicted to be a general rule by Colowick et al. (1966) and shown to be correect for evolutionarily divergent sources of L-lactate dehydrogenase and malate dehydrogenase, both A-stereospecific enzymes (Arnold et al., 1976).

NMR experiments using the chromosomal DHFR and [4-^{2}H]NADPH have previously shown that hydride ion transfer is to the C6 *si* face of the pteridine ring in dihydrofolate, producing the biologically active isomer of tetrahydrofolate (Pastore & Friedkin, 1962). Under the growth conditions

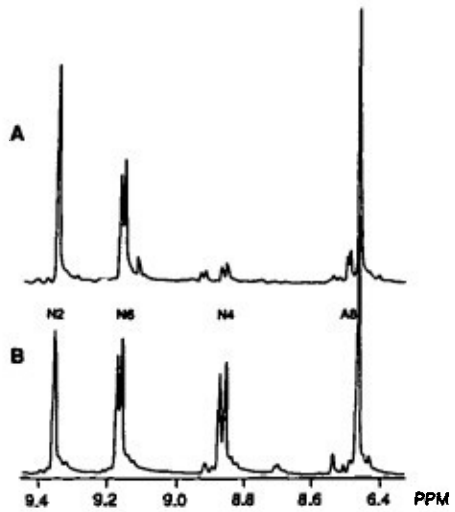

FIGURE 5: Comparison of the aromatic region of the 500-MHz ^1H NMR spectrum of (A) $NADP^+$ isolated from the oxidation of [4-(*R*)-^1H,4(*S*)-^2H]NADPH by RBG200 DHFR and (B) commercial $NADP^+$. The nicotinamide protons are labeled N2, N6, and N4. The adenine H8 proton is labeled A8.

utilized, *E. coli* strain C600 will not grow in the presence of trimethoprim without the plasmid carrying the gene for RBG200 DHFR (Vermersch et al., 1986). Therefore, the only source of tetrahydrofolate in the presence of trimethoprim is that synthesized by the type II DHFR encoded by the plasmid RBG200. Since the tetrahydrofolate synthesized by RBG200 DHFR is necessary for cell growth and only the C6 *si* isomer of tetrahydrofolate is biologically active (Charlton et al., 1979; Fontecilla-Champs et al., 1979), RBG200 DHFR is transferring the C4 *pro-R* hydrogen of NADPH to the C6 *si* face of the pteridine ring in dihydrofolate.

Evidence for Two Interconverting RBG200 DHFR–$NADP^+$ Complexes in Solution. Figure 6 shows the nicotinamide HN2 and HN6 protons of 5 mM $NADP^+$ in the presence of 0.5 mM RBG200 DHFR as a function of time at 25 °C. The presence of enzyme caused extensive broadening and upfield chemical shifts of both nicotinamide resonances (Figure 6B). Chemical shift changes and line broadening for the adenine and ribose resonances of $NADP^+$ were also observed immediately upon addition of RBG200 DHFR (data not shown). A second set of nicotinamide proton resonances were also observed downfield of the exchange-broadened resonances having chemical shifts closer to those of the free coenzyme (Figure 6B,E). After incubation of $NADP^+$ with RBG200 DHFR at 25 °C for 1 h, two distinct sets of HN2 and HN6 proton resonances are more clearly observed (Figure 6C). Continued incubation for 10.5 h results in the observation of just a single set of HN2 and HN6 proton resonances (Figure 6D) with chemical shifts closer to those of the free coenzyme (Figure 6E). This behavior is consistent with the slow time-dependent conformational interconversion between two distinct binary RBG200 DHFR–$NADP^+$ complexes. In the initial conformation, conformation I, the nicotinamide portion of the coenzyme is in an environment which produces upfield chemical shifts of 0.021 and 0.049 ppm in the HN2 and HN6 protons, respectively. The chemical shift changes in the nicotinamide proton resonances indicate that the oxidized nicotinamide ring does interact with RBG200 DHFR in a highly specific manner. In contrast, the chemical shift changes on coenzyme binding to the final binary conformation, conformation II, are smaller, 0.011 ppm, for both HN2 and HN6 and downfield from the resonances of the free coenzyme. The line broadening of the HN2 and HN6 resonances in conformation I, compared with

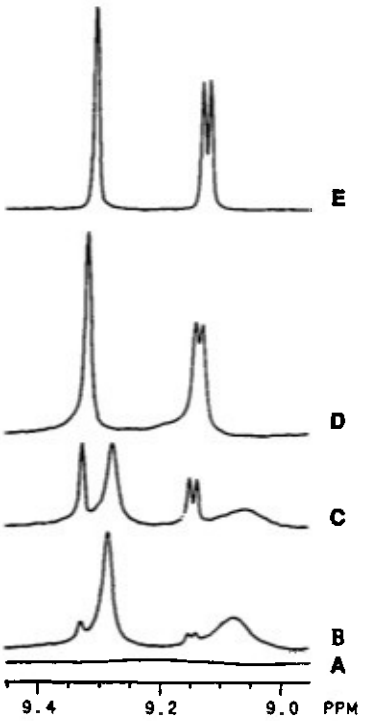

FIGURE 6: HN2 and HN6 proton resonances of 5 mM $NADP^+$ in the presence of 0.5 mM RBG200 DHFR at 25 °C showing the time-dependent interconversion from the initial binary RBG200 DHFR–$NADP^+$ complex (conformation I) to the final binary complex (conformation *II*). (A) Aromatic proton region of RBG200 DHFR in the absence of $NADP^+$; (B) nicotinamide HN2 and HN6 protons of 5 mM $NADP^+$ immediately after addition of 0.5 mM RBG200 DHFR; (C) proton NMR spectrum of the aromatic region after 1-h incubation with RBG200 DHFR showing two sets of HN2 and HN6 proton resonances of the coenzyme; (D) proton NMR spectrum of the aromatic region after 10.5-h incubation with RBG200 DHFR showing the HN2 and HN6 protons of the coenzyme in the final binary RBG200 DHFR–$NADP^+$ complex; (E) nicotinamide HN2 and HN6 protons of free $NADP^+$ in solution under identical conditions. NMR spectra were obtained at 500 MHz by use of 32 transients with 16K data points, a spectral width of 7042 Hz, a recycle time of 3 s, and a line broadening of 2 Hz.

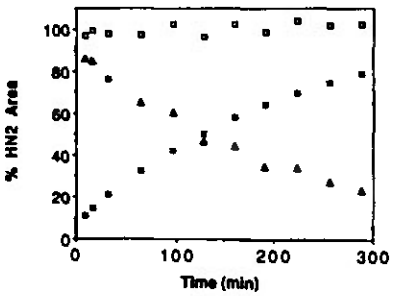

FIGURE 7: Kinetics of interconversion at 25 °C of RBG200 DHFR–$NADP^+$ conformation I to conformation II by monitoring of the integral of nicotinamide HN2 resonance in both complexes. The reaction contained 5 mM $NADP^+$ and 0.5 mM RBG200 DHFR in 50 mM potassium phosphate buffer in ^2H_2O, pH 5.9. Area of the HN2 resonance in conformation I (△). Area of the HN2 resonance in conformation II (■). Sum of the area of HN2 in both conformation *I* and conformation *II* (□).

that in conformation II, implies that the dissociation of $NADP^+$ in conformation I is slower than in conformation II. The rate of interconversion of conformation I to conformation II was determined by measuring the area of the HN2 resonance in both conformations I and II as a function of time (Figure 7). Figure 7 shows the decrease in area of the HN2 resonance in conformation I with the corresponding increase in area of the HN2 resonance in conformation II. The total intensity of the HN2 resonance from both conformations I and

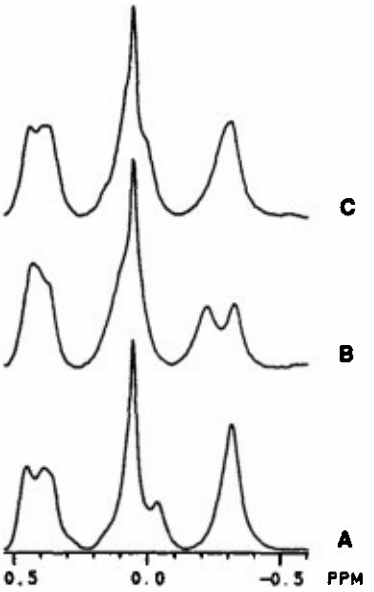

FIGURE 8: NMR spectra of the upfield-shifted methyl resonances in RBG200 DHFR showing the effect of $NADP^+$ addition and the time-dependent interconversion from conformation I to conformation II. The sample contained 0.5 mM RBG200 DHFR in 50 mM potassium phosphate buffer in ^2H_2O at pH 5.9. (A) Upfield-shifted methyl region of RBG200 DHFR in the absence of $NADP^+$. (B) Upfield-shifted methyl region of RBG200 DHFR 9 min after the addition of 5 mM $NADP^+$. (C) Upfield-shifted methyl region of RBG200 DHFR 10.5 h after the addition of $NADP^+$. NMR spectra were obtained under the conditions described in Figure 6.

II remained constant throughout the time course of tbe interconversion, indicating that a two-state model of the interconversion process is adequate (Figure 7). From the data in Figure 7, an apparent first-order rate constant of 1.0×10^{-4} s^{-1} at 25 °C was found for the interconversion of conformation I to conformation II. At 5 °C an apparent first-order rate constant of 9.3×10^{-6} s^{-1} for this interconversion was determined (data not shown). From the apparent first-order rate constants, an energy of activation E_a = 20 kcal/mol was estimated. The activation rate parameters estimated for the interconversion are $\Delta H^\ddagger$ = 33 kcal/mol, $\Delta S^\ddagger$ = 34 cal/(mol·deg), and $\Delta G^\ddagger$ = 23 kcal/mol (at 25 °C). These values are in the range of those observed for tbe conformational interconversion of two distinct cbromosomal *Lactobacillus casei* DHFR–trimethoprim–$NADP^+$ ternary complexes (Gronenborn et al., 1981a,b).

The binding of $NADP^+$ and the formation of the initial binary complex (conformation I) can also be detected by monitoring the chemical shifts of the upfield-shifted methyl resonances of RBG200 DHFR (Figure 8). Comparison of the upfield-shifted methyl regions of RBG200 DHFR in the absence of (Figure 8A) and immediately after addition of $NADP^+$ (Figure 8B) reveals several chemical shift changes in the methyl resonances upon addition of coenzyme and formation of binary conformation I. After 10.5 h, time sufficient for complete interconversion of conformation I to conformation II, a different pattern of chemical shifts for the methyl resonances is observed for the complex in conformation II (Figure 8C). This pattern is more similar to that found for enzyme alone. However, the line widths of the methyl resonances are broader than those found for enzyme alone due to an exchange contribution from the binding of $NADP^+$. Although conformational changes in the protein observed upon interconversion of the two complexes cannot yet be described in detail, alterations in the upfield-shifted methyl protons are most simply explained by altered geometric relationships of the methyl groups to aromatic rings of either the protein or coenzyme. Cbemical shift changes observed in this region are consistent with the formation of an initial binary RBG200 DHFR–$NADP^+$ complex, involving an initial conformational alteration in the protein, which slowly converts to a second binary complex in which the coenzyme or the protein exists in a different environment or conformation. The existence of two or more conformational states has been detected in ternary complexes of chromosomal *L. casei* DHFR–trimethoprim–$NADP^+$, but not in binary complexes with $NADP^+$ (Hyde et al., 1980; Gronenborn et al., 1981a,b).

Conclusions. A rapid and gentle purification procedure for RBG200 DHFR, a cloned and overproduced derivative of R388 DHFR having the sequence Thr-Thr-Ser-Arg-Thr-Leu at tbe carboxy terminus, has been developed wbich yields milligram quantities of apparently homogencous protein witb a specific activity 1.5-fold greater than that previously reported for the R388 protein (Amyes & Smith, 1976). The active form of RBG200 DHFR was found to be a tetramer in solution with a pH optimum near 5.9. RBG200 DHFR, a type II dihydrofolate reductase, has been shown to stereospecifically transfer the *pro-R* proton of NADPH to dihydrofolate, making it a member, along with chromosomal dihydrofolate reductases, of the A-stereospecific class of dehydrogenases. NMR studies monitoring the proton chemical shifts of $NADP^+$ upon addition of RBG200 DHFR have permitted the detection of two distinct binary RBG200 DHFR–$NADP^+$ complexes in solution. The nature of the conformational states and their structural/functional role in catalysis remain to be determined. Studies are in progress to elucidate the structural differences between these two complexes in order to better understand their role in coenzyme binding and activity. The ability to rapidly prepare large quantities of RGB200 DHFR will facilitate physical and structural studies aimed at understanding the unique mode of cofactor interaction with this novel dihydrofolate reductase.

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Stability of mutant type II dihydrofolate reductase proteins in suppressor strains

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Summary

In the course of study of a (Glu-58 to Gln-58) mutant type II dihydrofolate reductase (DHFR), it was found that the altered DHFR was poorly produced in vivo. Investigations with several common laboratory *Escherichia coli* strains including *htpR* and *lon* strains bearing plasmids expressing the Gln-58 DHFR indicated a correlation of rapid degradation with the presence of a *sup*$^+$ phenotype. The *sup*^{o} strain MC1061(p3) was transformed with a series of plasmids containing the Gln-58 DHFR gene with and without an additional *supF* gene, and expression levels were compared. The *supF*$^+$ constructs exhibited little accumulation of the Gln-58 DHFR, while reasonable levels were found in the *sup*^{o} cases. Experiments with extracts of plasmid-free *sup*$^+$ and *sup*^{o} strains showed rapid degradation by certain strains compared to MC1061(p3) and this degradation was not dependent upon ATP.

In another route to increasing the stability of labile DHFR derivatives, mutagenesis of a strain bearing a N-terminally shortened Gln-58 DHFR was performed. Selection and analysis of a trimethoprim-resistant stable mutant showed that this DHFR gene contained a triple repeat of leu-pro-ser in the enzymatically non-essential N-terminal portion of the protein.

Production; Mutagenesis; Degradation; N-terminus; Stress; Enzyme

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Introduction

Even when protein synthesis from a recombinant gene has been completely optimized, poor yields may result due to instability of the protein product. Accumulation of a "foreign" protein, particularly if it is folded unnaturally may actually result in the induction of *Escherichia coli* protease genes (Goff and Goldberg, 1985). Instability of short foreign polypeptides in *E. coli* (Itakura et al., 1977) is a problem which can be overcome by forming fusions with larger proteins (Itakura et al., 1977; de Geus et al., 1987; Shen, 1984). Frequently, these abnormal proteins will be sequestered into insoluble aggregates or inclusion bodies. While inclusion bodies can afford some protection against proteases (Kitano et al., 1987), protein isolated from inclusion bodies can be difficult to renature (Hellebust et al., 1989). Another strategy for protecting a recombinant protein involves fusing the protein to something which will allow it to be transported to the periplasm; there are fewer proteases in the periplasm and the types of proteases may be different for a given strain within and outside the cell (Swamy and Goldberg, 1982). Rat preproinsulin fused to a bacterial signal sequence was 10-fold more stable in the periplasm of *E. coli* than in the cytoplasm (Talmadge and Gilbert, 1982).

There are protease level differences among *E. coli* strains and it is possible that a recombinant protein will be degraded at different rates in different strains. For example, recombinant Ada protein is degraded in *E. coli* B from a 39 kDa protein to 19 and 20 kDa fragments, whereas no significant degradation occurs in *E. coli* K12 (Bhattacharyya et al., 1988).

Addition of a protease inhibitor during the purification process can prevent or slow degradation, however, inhibitors are not always effective and they can complicate the procedure by inhibiting the activity of the protein (Hellebust et al., 1988). It can be more practical to use a strain which lacks one or more of the normal proteases. The *htpR* (*rpoH*) gene product is required for the induction of the heat shock proteins (Neidhardt and VanBogelen, 1981) including the *lon* gene product, protease La (Goff et al., 1984). Decreased degradation of abnormal or foreign proteins has been observed in *E. coli* strains that are deficient in *lon* or *rpoH* activity (Goff et al., 1984; Buell et al., 1985). Additive effects reported for *lon* and *rpoH* on somatomedin-C stability indicate that at least one other *rpoH*-regulated protease may be involved in the degradation of foreign proteins in *E. coli* (Buell et al., 1985).

In the course of making site-specific mutations in a gene for a type II dihydrofolate reductase (DHFR), we have observed some effects of the *E. coli* host strain and the N-terminus of the gene on the stability of the mutant proteins. Previously, it was found that many C-terminal additions to the protein yielded larger proteins which were both stable and enzymatically active; DHFR is a convenient carrier for foreign peptide segments (e.g. epitopes) added in this manner (Vermersch et al., 1986). The enzyme is also quite tolerant to alterations at the N-terminus. Protease digestion experiments have been described in which the first 15 amino acids of the wild-type R67 Type II DHFR were removed without abolishing activity in vitro (Matthews et al., 1986). Genetic experiments, in which the 5′ coding region for the

wild-type R388 enzyme was shortened, showed that N-terminal deletion derivatives of the DHFR could also function in vivo. An R388 *dhfr* gene, shortened by 21 base pairs (Vermersch and Bennett, 1987) and a totally synthetic *dhfr* mini-gene, shortened by 30 base pairs (Vermersch and Bennett, 1988) function in vivo as evidenced by their ability to confer resistance to trimethoprim (Tp^R). Trimethoprim is a folate analog to which type II DHFRs show exceptional resistance (Aymes and Smith, 1974).

The mini-DHFR may be isolated in relatively large quantities when the gene is over-expressed on high-copy plasmids (Vermersch and Bennett, 1988). A Gln was substituted for a Glu at position 58 (as numbered in the full-length enzyme) in the mini-DHFR to experimentally test the model for folate-binding proposed by Matthews et al. (1986). No activity was detected for the Gln-58 mini-DHFR in vivo as evidenced by the inability of *E. coli* D3-157 (*dhfr*$^-$) containing multi-copies of the mutant *dhfr* mini-gene to grow on *dhfr* selective media (Singer et al., 1985). DHFR activity is not lost, however, when the Gln-58 mutation is present on the full length gene.

Although the full length Gln-58 *dhfr* gene conferred a Tp^R, *dhfr*$^+$ phenotype, very little of the enzyme could be recovered using standard *E. coli* strains and DHFR purification procedures (Vermersch et al., 1986). Singly, neither the N-terminal truncation nor the Gln-58 substitution abolishes DHFR activity; however, each mutation destabilizes the DHFR to some extent. The effects of these mutations are additive, as evidenced by the inability of the Gln-58 mini-DHFR to confer the Tp^R, *dhfr*$^+$ phenotype. This study utilizes these proteolytically sensitive DHFRs to investigate attributes of the host strain and the DHFR structure which contribute to the enzyme's degradation in *E. coli*.

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The genotypes of bacterial strains are presented in Table 1. Transformation and selection were as described (Russell and Bennett, 1982) except that temperature sensitive strains were transformed and plated at 32°C. For in vivo experiments, strains were grown in Mueller-Hinton media containing 50 mg l^{-1} Tp from individual colonies maintained on Mueller-Hinton Tp agar plates. For analysis of DHFR protein levels in the cells, aliquots of the cultures were taken, lysed, and subjected to SDS-polyacrylamide gel electrophoresis as described (Vermersch et al., 1986 and Vermersch and Bennett, 1988). Details of particular experiments are given in the figure legends.

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TABLE 1

Bacterial strains

Strain	Genotype	Gln-58 DHFR production [a]	Source/Reference
CAG629	lac_{am} trp_{am} pho_{am} $SupC^{ts}$ *rpsL* *mal lon*$^-$ $htpR_{165}$ Tn10	+ +	Baker et al., 1984; Gottesman, 1990
$W3350_{ts}$	$htpR_{3350}$ *gal sup*^{o}	+ + +	Neidhardt and VanBogelen, 1987
MC1061(p3)	Δ(*lac*)74 *galU galK* $strA^R$ *hsdR* Δ(ara, leu) Ap_{am} Tc_{am}	+ + +	Seed, 1983
71-18	Δ(lac-pro)F′ $laci^q$ lacZΔM15 pro$^+$ *supE*	+	Dente et al., 1983
D3-157	F$^+$ *guaB22 rpsL*125 fol-200	not tested	Singer et al., 1985
KL451	F′ *trpA* (UAG211)/*metB glyV*55 Δ(*tonB-trpA*E15)	+	Murgola, 1985
KL1270	F′ *trpA* (UAF211)/*metB glyU* (*SupU*AG) *glyV*55 Δ(*tonB-trpA*B)	+	Murgola, 1985
KL1446	F′ *trpA* (UAA234)/*metB glyV* (*SupU*AA/G) *glyV*55 Δ(*tonB-trpA*B)	neg.	Murgola et al., 1984
FTP4178	F′ *trpA* (UAG211)/*glyV*55 *rpoB* Δ(*tonB-trpA*B17)	+	Murgola, 1985
FTP4223	F′ *trpA* (UAG211)/*glyT*(*SupU*AA/G) $argE_{am}$ *glyV*55 *rpoB* Δ(*tonB-trpA*B)	neg.	Murgola et al., 1984
FTP4226	F′ *trpA* (UAG211)/*glyT*(*SupU*AG-8) $argE_{am}$ *glyV*55 *rpoB* Δ(*tonB-trpAB*)	neg.	Murgola et al., 1983

[a] + + +, high accumulation of gln-58-DHFR when bearing pPVΔBSP2; + +, medium expression; +, low to medium expression; neg., the plasmid was selectable but no DHFR band was observed on gels.

listed in Table 2. pPV9$^+$214 is a derivative of pPV9$^+$SYN, containing a semi-synthetic, Tp^R *dhfr* gene (Vermersch and Bennett, 1988). It contains a synthetic N-terminal DHFR coding fragment (*Bam*HI/*Ban*I) from pPV214SYN2, and a core DHFR coding fragment derived from pPVL22-20 (*Ban*I/*Xho*I). The synthetic C-terminal DHFR coding sequence (*Xho*I/*Eco*RI) and remaining vector elements are those of pPV9$^+$SYN. The dhfr-containing plasmids from which pPV9$^+$214 was made have been described previously (Vermersch and Bennett, 1988). pPV9$^+$214-1 is identical to pPV9$^+$214 except that it contains a codon change of GAG (Glu) to CAG (Gln) at position 58 of the amino acid sequence. The substitution was made by oligonucleotide mutagenesis (Kunkel, 1985) and confirmed by DNA sequence analysis using the Sequenase kit supplied by the United States Biochemical Corporation. Descriptions of the plasmids can be found in Table 2. Plasmid pPV224 was formed by inserting the *SupF* gene carried on an *Eco*RI fragment of pVX (Seed, 1983) into *Sma*I and *Hin*dIII cleaved pRBG215 (Vermersch et al., 1986) after

TABLE 2

Plasmid properties

Plasmid	Encoded DHFR	Genetic markers	Copy number	Promoter
pPV9$^+$214	Δ10aa N-term.	Tp^R Ap^R	medium [a]	tac [c]
pPV9$^+$214-1	Δ10aa N-term. Gln-58	Tp^S Ap^R	medium [a]	tac [c]
pPV224-1-1	wild-type full N-term. Gln-58	Tp^R *SupF*	high [b]	dhfr
pPVΔBSP2	wild-type full N-term. Gln-58	Tp^R	high [b]	dhfr
pPVBSP2-sup	wild-type full N-term. Gln-58	Tp^R *SupF*	high [b]	dhfr
pPV224-2	wild-type	Tp^R *SupF*	high [b]	dhfr
pMT134-sup	wild-type *dhfr* interrupted by SupF	Tp^R *SupF* Ap^R	medium [a]	dhfr
pMT134	wild-type *dhfr* interrupted by *Bam*HI linker	Tp^R Ap^R	medium [a]	dhfr
pRBG200	+6aa at C-term.	Tp^R	high [b]	dhfr

[a] These plasmids bear the basic replication origin of pBR322 (Swift et al., 1981; Vermersch and Bennett, 1988); [b] These plasmids bear an origin of replication which is derived from the high copy number plasmid pRBG156 (Gayle et al., 1986); [c] Ammann et al., 1983.

making all ends blunt by reaction with the large fragment of DNA polymerase I. The derivative, pPV244-1, bore a unique *Eco*RI site at the C-terminus of the *dhfr* gene formed by inserting an *Eco*RI linker into the *Pvu*II site of pPV224. *Ban*I/*Eco*RI fragments of pPV9$^+$214-1 and pPV9$^+$214 were inserted into pPV224-I to form pPV224-1-1 and pPV224-2, respectively.

Purificotion of protein

pRBG200 DHFR (Vermersch et al., 1986) and pPVΔBSP2 (Gln-58) DHFR were prepared from MC1061(p3) bearing the appropriate plasmid as described (Vermersch et al., 1986).

Preparation and use of cell extrocts

For in vitro degradation experiments, *E. coli* strains were grown aerobically in LB media to $A_{600} = 0.4$. The culture (200 ml) was pelleted, washed with phosphate

buffer (0.01 M potassium phosphate, pH 7.6), resuspended in 9 ml potassium phosphate buffer and sonicated (8–30 s pulse at 50% duty cycle, amplitude 7) using a Heat Systems-Ultrasonics W-225 sonicator with a 1/2″ tip. The sonicate was centrifuged at 20,000 × *g* for 30 min to pellet cell debris and the supernatant was removed and used as the extract in the protein degradation experiments. Protein concentration was determined by the method of Bradford (1976).

Degradation assays contained 10 mg Gln-58 DHFR, cell extract containing 3.8 mg protein, and 2 ml of 10 mM ATP in a total volume of 20 ml potassium phosphate buffer, pH 7.6. The samples were incubated 0–40 min at 37°C, SDS sample buffer (10 ml) was added, and the solution was boiled for 5 min prior to loading on a 15% SDS-polyacrylamide gel. After electrophoresis the gel was stained with Coomassie blue and photographed. With specified extracts, ATP and other small molecules were removed by three cycles of resuspension and centrifugation in a Millipore 10,000 MW cutoff Ultrafree-MC filter unit. After this treatment the ATP level was determined using the luciferase-luciferin assay as recommended by the supplier (Sigma Chemical Co.) and found to be below 10^{-10} M.

Random mutagenesis

Random mutagenesis of pPV9^{+}214-1 in *E. coli* 71-18 was conducted in the following manner. A 5 ml culture of *E. coli* 71-18 cells containing pPV9^{+}214-1 was grown overnight in LB plus 30 mg ml^{-1} ampicillin (Ap). The culture was plated (0.2 ml) onto Mueller Hinton agar containing Tp and 40 mg ml^{-1} isopropyl-1-thio-β-D-galactoside (IPTG). A large crystal of N-methyl-N′-nitro-N-nitrosoguanidine was placed on the agar in the center of the plate and the plates were incubated at 37°C for 2–3 days. The mutagenesis produced a large number of Tp^{R} colonies. DNA was isolated from several colonies and analyzed by digestion with *Ava*II; a Glu-58 (...GGTCGAG...) to Gln-58 (...GGTCCAG...) mutation in the *dhfr* gene results in the creation of a unique *Ava*II site (GGACC or GGTCC). The DNA from some of the colonies which did not show a loss of the *Ava*II site was used to re-transform *E. coli* 71-18. This step served to establish that the conferred Tp^{R} was due to a mutation in the plasmid *dhfr* gene (other than a reversal of the Gln-58 mutation) and not due to a mutation in a chromosomal gene of the strain.

Results

When the Gln-58 DHFR was poorly recovered from preparations that followed the pRBG200 DHFR purification protocol, steps were taken to increase Gln-58 DHFR accumulation. The effect of the host strain was studied; different strains which usually express recombinant proteins well and strains with *lon* or *htpR* mutations were used. Subsequently, various plasmid constructs bearing the gene were then studied in an attempt to increase DHFR levels.

In vivo expression studies

The ability of several strains to produce the mutant DHFR was explored by transformation with pRBG200 (Glu-58), pPV224-2 (Glu-58), or pPVΔBSP2 (Gln-58). Table 1 includes the strains transformed and denotes the relative accumulation of Gln-58 DHFR. The Gln-58 DHFR was produced in quantity only in certain strains, while Glu-58 DHFR was produced rather well in all these strains. *Sup*$^-$ strains MC1061, KL451, and FTP4178 showed accumulation of the mutant DHFR, while *sup*$^+$ strains 71-18, KL1270, and FTP4226 revealed little detectable DHFR, although the plasmid could still be selected as Tp^R in those strains and the cultures were grown under selection for Tp^R. Evidently only a small amount of type II DHFR is sufficient to render the cells Tp^R. This is consistent with the low level of DHFR found in the original R388 or R67 bearing strains (Zolg et al., 1978; Smith et al., 1979).

In vitro degrodation

In order to evaluate the ability of various strains to degrade the Gln-58 DHFR in the absence of plasmid-related phenomenon, extracts from log phase cultures of selected strains were prepared and the ability of the extracts to degrade purified Gln-58 DHFR was studied. Extracts isolated from MC1061(p3) degraded purified Gln-58 DHFR slowly but yielded no degradation of pRBG200 DHFR under similar conditions. The more rapid degradation of Gln-58 DHFR was found in all of the strains tested. Fig. 1A shows a typical gel for pRBG200 vs Gln-58 DHFR degradation with extracts from KL1270. The rate of degradation in the other strains was higher than in MC1061(p3); an example is shown in Fig. 1B.

Since ATP hydrolysis is required for certain proteases (e.g. *lon*), the effect of ATP on the degradation of Gln-58 DHFR in FTP4226, a strain representative of those which did not allow accumulation of the Gln-58 DHFR, was examined. Fig. 2 shows the similar degradation noted in extracts with 1 mM ATP added vs extracts without additional ATP. In addition, no significant difference was observed in extracts dialyzed to remove small molecules (ATP level $< 10^{-10}$ M).

Mutotions of lon *and* rpoH

Strains with mutations in *lon* or *rpoH* (htpR) are often used to allow accumulation of labile foreign proteins, therefore, the ability of *lon* and *rpoH* strains to accumulate the mutated DHFR protein was examined. CAG629 and W3350$_{ts}$ were transformed with pPV224-2 (Glu-58, *supF*$^+$), pPV224-1-1 (Gln-58, *supF*$^+$) and pPVΔBSP2 (Gln-58, *supF*^{o}). The *htpR*$_{165}$ gene of CAG629 contains an amber mutation which is partially suppressed by the tRNA product of the chromosomal *supC*^{ts} gene at low temperature. At 42° C suppression of the amber mutation by the temperature sensitive chromosomal suppressor no longer occurs. However, in strains bearing a *sup*$^+$ plasmid the strain would be phenotypically *htpR*$^+$*lon*$^-$ at both temperatures and would serve as a *lon*$^-$ strain. In W3350$_{ts}$, the *htpR*$^-$ phenotype is

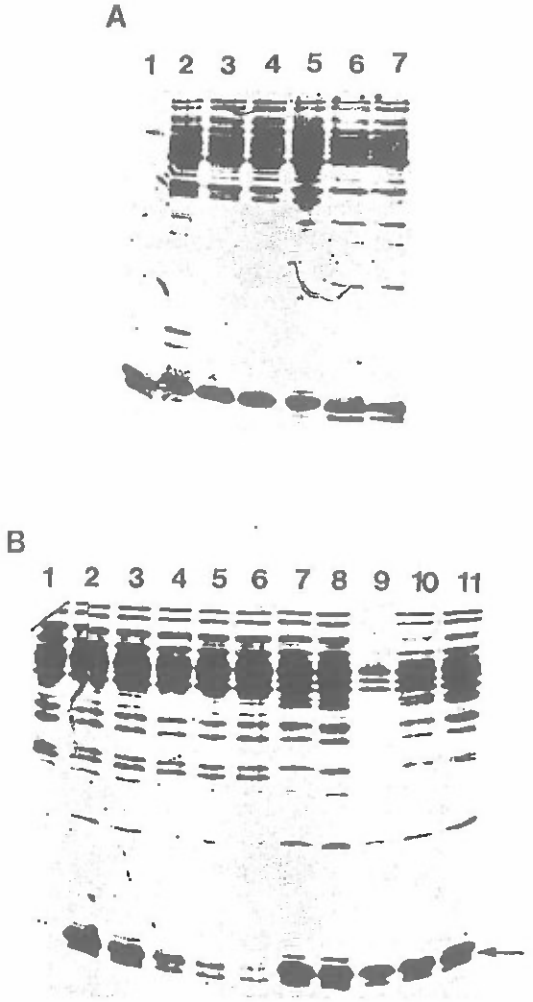

Fig. 1. SDS-PAGE of in vitro degradation samples. (A) Degradation of pRBG200 DHFR vs Gln-58 (pPVΔBSP2) DHFR in extracts of KL1270. Lane 1, pRBG200 DHFR only. Lanes 2–4 are extract incubated with pRBG200 DHFR for 0, 10 and 20 min, respectively. Lanes 5–7 are extract incubated with Gln-58 (pPVΔBSP2) DHFR for 0, 10, and 20 min, respectively. (B) Degradation of Gln-58 (pPVΔBSP2) DHFR in two *E. coli* strains. Lane 1 is FTP4226 extract, lanes 2–6 are FTP4226 extract incubated with Gln-58 DHFR for 0, 10, 20, 30 and 40 min, respectively. Lanes 7–11 are MC1061(p3) extract incubated with Gln-58 DHFR for 0, 10, 20, 30 and 40 min, respectively. Arrows designate migration of the DHFR monomers on the two gels.

not due to a suppressible mutation but is *htpR*$^-$ because the *htpR* gene contains a missense mutation which renders the sigma factor encoded by *htpR* inactive at higher temperatures; therefore, at high temperature this would serve as an *htpR*$^-$ strain.

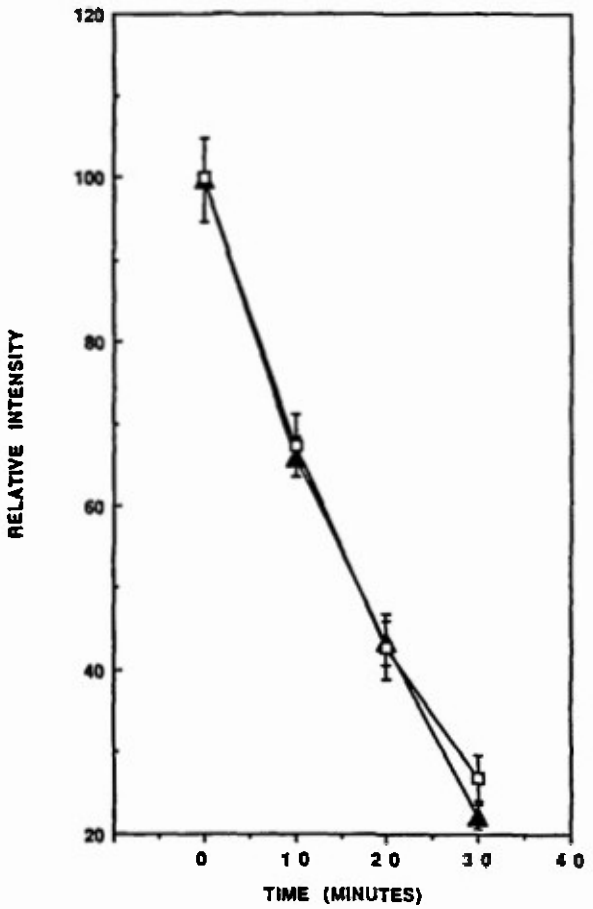

Fig. 2. The effect of ATP on degradation of Gln-58 DHFR by extracts of FTP4226. The in vitro degradation experiment was conducted as described in Methods. After staining, gels were photographed and analyzed by densitometry. Intensity of bands was compared to zero time sample. Extract without ATP: □; extract with ATP: ▲; bars indicate standard error.

The DHFR production in each culture was analyzed by SDS-polyacrylamide gel electrophoresis. The finding was that $W3350_{ts}$ yielded high production of Gln-58 DHFR at 32°C and 43°C from the *sup*° DHFR plasmid, pPVΔBSP2, while low amounts were found with the *sup*$^+$ DHFR plasmid, pPV224-1-1. CAG629 also produced significant (but less than $W3350_{ts}$) quantities of Gln-58-DHFR from the *sup*° plasmid, but again less DHFR was found in the corresponding *sup*$^+$ plasmid strain. Thus it did not seem that the presence of an *htpR*$^-$ or *lon*$^-$ mutation necessarily conferred high production capability for the Gln-58 DHFR when the altered DHFR gene was carried on *sup*$^+$ plasmid.

To further study production of mutant DHFR in $W3350_{ts}$ and compare it with MC1061(p3), a *rpoH*$^+$, *lon*$^+$, *sup*° strain, these two strains bearing plasmids

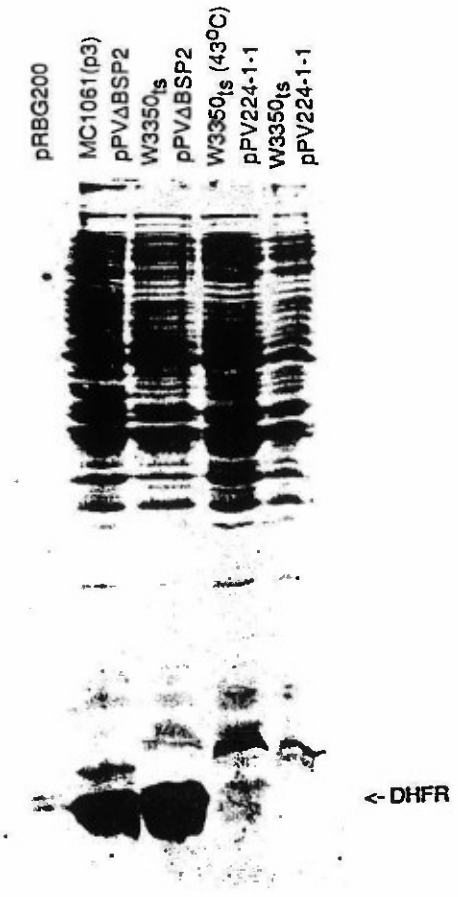

Fig. 3. A comparison of Gln-58 DHFR production in $W3350_{ts}$ and MC1061(p3). A Coomassie blue-stained 12% SDS-polyacrylamide gel shows extracts from cultures of $W3350_{ts}$ and MC1061(p3) bearing pPVΔBSP2 or pPV224-1-1, as indicated. Transformed cells in 5 ml of Mueller-Hinton plus Tp were grown at 37° C with shaking to an A_{650} of approx. 0.4, divided into two tubes (2.5 ml each) and allowed to grow 3 h more either maintained at 37° C or in one case ($W3350_{ts}$/pPV224-1-1, as indicated) shifted to 43° C. A partially purified preparation of the DHFR encoded by pPRBG200 has been included as a protein size standard (85 aa). Arrow designates the migration of the DHFR monomers.

pPV224-1-1 (*sup*$^+$, Gln-58 DHFR) and pPVΔBSP2 (*sup*°, Gln-58 DHFR) were examined (Fig. 3). Perhaps slightly more DHFR was present in the $W3350_{ts}$pPV224-1-1 culture shifted to 43° C than was present in the culture maintained at 37° C; however, overwhelmingly more DHFR was present in both MC1061(p3) and $W3350_{ts}$ containing pPVΔBSP2. The results of this experiment suggest that the ability to accumulate the pPVΔBSP2-encoded Gln-58 DHFR is not a function of *htpR* or *lon*, but can possibly be correlated with *supF*.

The role of the suppressor gene

An experiment was conducted to examine the possible role of the suppressor tRNA gene in the intracellular accumulation of the Gln-58 DHFR and to try to eliminate specific plasmid effects. Production of DHFR was compared in *sup*° MC1061(p3), a strain exhibiting relatively low degradation of the Gln-58 DHFR, carrying the following plasmids: (1) pPVΔBSP2 (Gln-58) cotransformed with a

Fig. 4. The effect of amber suppressors on the accumulation of the Gln-58 DHFR in *E. coli*. A Coomassie blue-stained 12% SDS-polyacrylamide gel shows whole cell extracts from overnight cultures of MC1061(p3) (*sup*°) or 71-18 (*supE*) transformed with the indicated plasmids. Arrow designates migration of the DHFR monomers.

pBR322-derivative (pMT134-sup) containing a cloned copy of the *supF* gene; (2) pPVΔBSP2-sup, (in which a copy of the *supF* gene had been cloned into the *Bam*HI site of pPVΔBSP2); (3) pPV224-1-1; (4) pPVΔBSP2. In addition, DHFR production was compared in the *supE* strain 71-18, transformed with pPVΔBSP2. Results of SDS-polyacrylamide gel analysis of whole cell extracts from cultures of these transformed cells are shown in Fig. 4. DHFR levels in extracts of transformed MC1061(p3) cells are highest with pPVΔBSP2 alone. pPVΔBSP2-sup and pPV224-1-1 levels are similar but lower. In contrast to the MC1061(p3) results, the DHFR levels in pPVΔBSP2-transformed 71-18 cells are low. The results of this experiment suggest that the level of the Gln-58 DHFR in the various cell extracts can be correlated with the presence of an amber suppressor. Also, more of the mutant DHFR was found in cells containing the *supF* gene on a pBR322-type plasmid than on a high-copy pRBG215-type (either recloned on pPVΔBSP2 or on the parent vector, pPV224-1-1). This could perhaps be explained by a difference in copies of *supF*.

Other differences between pPVΔBSP2 and pPV224-1-1 were sough. which could be responsible for the observed difference in DHFR production. Because the Glu-58 to Gln-58 mutation produces a new *Ava*II site in the *dhfr* gene, it was known throughout these experiments that the original mutation was still present in the pPVΔBSP2 gene, however, the possibility existed that another mutation in the protein was producing compensatory stabilizing effects. The *dhfr* structural gene from pPVΔBSP2 was cloned into pEMBL9$^+$ and sequenced. No secondary mutations were found in the structural gene. Since pPVΔBSP2 was formed from the *sup*$^+$ plasmid pPV224-1-1 and since pPVΔBSP2-sup (prepared from pPVΔBSP2) exhibited the same effect as pPV224-1-1, the possibility that the difference in stability is due to something other than the *sup* gene on the plasmid seems unlikely.

Introducing compensatory changes into a mutant DHFR gene

A totally different approach to stabilizing the mutant DHFRs would involve the purposeful introduction of compensatory changes in the protein which counteract the destabilizing effect of the original mutation and thereby render it less susceptible to proteolytic attack. For the purpose of this investigation it would be necessary to modify the *dhfr* in a way that stabilizes the protein without tampering with the residues involved in the phenomenon under analysis.

It was recognized that the Gln-58 mini-DHFR encoded by pPV9$^+$214-1 might be useful in the elucidation of structural factors important to the stability and function of the DHFR. One advantage of using the Gln-58 mini-DHFR is that a phenotypic selection exists for functional revertants. This DHFR does not confer Tp^R because of the additive effects of the Glu-58 to Gln-58 mutation and the shortened N-terminus. Either of the modifications present singly in an otherwise wild-type DHFR does not destroy the conferred Tp^R. Any strengthening of the forces required for the maintenance of the functional form of the enzyme may, therefore, be detected by a reversion to the Tp^R phenotype. Since neither the full N-terminus nor the Glu-58 residue was absolutely required for function it was, therefore, at least

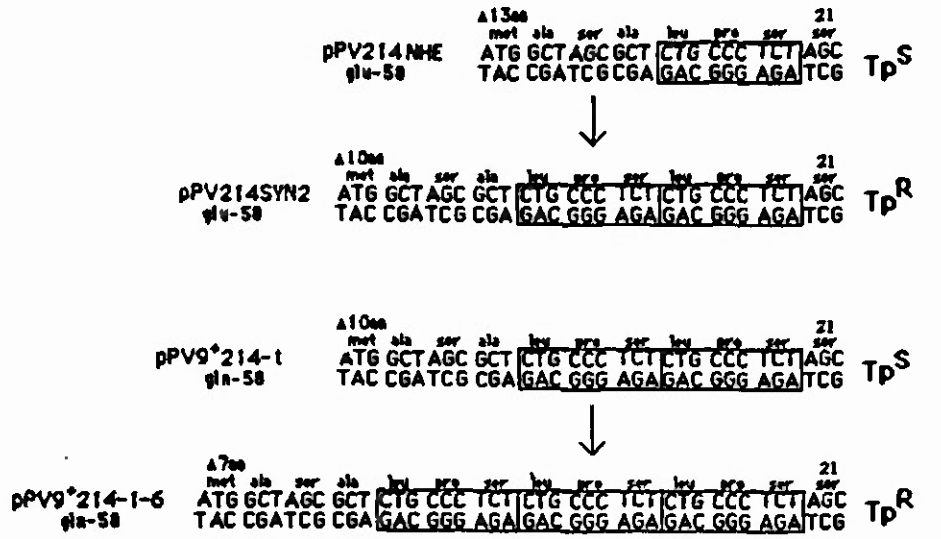

Fig. 5. The effect of successive N-terminal additions of the leu-pro-ser triplet on DHFR function in vivo. The 9 bp sequence corresponding to the leu-pro-ser triplet is underlined. The ability of each of the plasmids to confer resistance to trimethoprim (Tp^R or Tp^S) is indicated. The amino acid residue encoded at position 58 (glu or gln) is shown for each of the plasmids.

theoretically possible to isolate second-site revertants. True revertants, those that exactly reverse the original mutation, would be expected to exist as a Tp^R background in the experiment. These plasmids would most likely be from Gln-58 back to the Glu-58. Because the Gln-58 mutation produces a new *Ava*II site in the *dhfr* gene this reverse mutation would be easily revealed by DNA restriction analysis.

Random mutagenesis of pPV9$^+$214-1 in 71-18 was performed as outlined in Methods. Sequence analysis of DNA from three Tp^R colonies revealed that the *dhfr* genes still contained the Gln-58 mutation, but also contained identical compensatory modifications at the N-terminus. In all three plasmids a duplication of 9 base pairs in the N-terminus segment had occurred (Fig. 5). This 9 residue duplication in the DNA corresponds to a 3 amino acid extension (leu-pro-ser) at the N-terminus of the mini-DHFR. This Tp^R plasmid encoding the Gln-58 DHFR with the modified N-terminus (Δ7aa), has been designated pPV9$^+$214-1-6.

It was previously shown with the construction of pPV224-1-1 that returning the full wild-type N-terminus to the Gln-58 mini-DHFR will restore Tp^R (Table 2). The result of this reversion analysis indicates that the N-terminal addition of a third copy of the leu-pro-ser triplet to the N-terminus mini-DHFR can also suffice to restore Tp^R.

Discussion

To experimentally test the model for folate binding proposed by Matthews et al. (1986), a mutation was made at position 58 in a *dhfr* mini-gene: Glu-58 to Gln-58.

This mutation made in the putative folate binding site of the N-terminally shortened DHFR appeared to destabilize the enzyme as evidenced by the very reduced levels of DHFR protein detectable by gel electrophoresis in extracts of cells expressing the mutated gene. In general, *sup*$^-$ *E. coli* strains showed an accumulation of the DHFR, whereas *sup*$^+$ strains showed little. However, there were variations in levels of accumulation among *sup* strains. Incubation of cell extracts of various *E. coli* strains with purified pPVΔBSP2 DHFR (Gln-58) resulted in a wide variation in the extent of degradation (Fig. 1). The presence of ATP did not appear to affect the degradation in strain FTP 4226; however, the degradation occurring in the strains bearing high copy numbers of *supF* were not directly tested in this assay. A set of isogenic strains was used in in vitro degradation experiments to try to further correlate the level of suppression with tbe relative rate of degradation of the Gln-58 DHFR. Even though gels were analyzed by densitometry, the variation in rate between strains compared to the sample measurement error did not allow a relationship to be observed. This may have been partially due to the relatively high background of degradation in this strain (compared to MC1061) and the relatively low level of suppression produced by the copy of the chromosomal or F$^+$ encoded *sup* gene.

It is possible that an effect of high plasmid copy number was producing stress on the cell, resulting in enhanced degradation in specific strains. Isolation of plasmid DNA from cultures bearing pPVΔBSP2, pPV224-1, pPV224-1-1, or pPVΔBSP2-sup revealed no dramatic difference in plasmid copy number or form, which was as expected for these related plasmids. The copy number of several of the high-copy number plasmids has been previously studied (Gayle et al., 1986). The high level of the suppressor produced by the high-copy number plasmid pPV224-1-1 appears to yield a pronounced increase in degradation activity which is especially noticeable in MC1061(p3), a strain with relatively low background degradation. A more successful expression system might include a low copy *sup*$^\circ$ plasmid with a strong promoter. A study by Peretti et al. (1989) suggests that, since RNA polymerase enzymes bind nonspecifically to DNA and adding plasmid copies means increasing the cellular concentration of nonpromoter sites where nonspecific binding can occur, a low copy number plasmid with a strong promoter would give the highest yield of recombinant protein. It may also be feasible to determine which specific *E. coli* protease is responsible for the degradation of the mutated DHFR, and to work with a strain which lacks that protease. This approach was used successfully with a recombinant fusion protein which was cleaved by the outer membrane protease OmpT (Hellebust et al., 1989).

Further mutagenesis of a Gln-58 N-terminally shortened *dhfr* gene resulted in duplication of 9 residues at the N-terminus, with a resumption of Tp^R (Fig. 5). The results of this experiment are reminiscent of those obtained during the development of the synthetic coding region for the shortened, but functional, N-terminus on the synthetic *dhfr* gene (Vermersch and Bennett, 1988). The three amino acid sequence, leu-pro-ser, is present as a single triplet in the mini-DHFR (Δ13aa at N-terminus) encoded by the Tp^S plasmid, pPV214NHE. In the construction of pPV214NHE an anomalous Tp^R product, pPV214SYN2, was obtained. The only difference between

Tp^S pPV214NHE and Tp^R pPV214SYN2 is that the latter plasmid encodes a DHFR which contains a duplication of the leu-pro-ser triplet at the N-terminus.

The N-terminus of the *dhfr* of pPV214SYN2 (Δ10aa) is also present on the Tp^R plasmid pPV9$^+$214. The ability of the pPV9$^+$214 encoded DHFR to confer Tp^R was destroyed by the Glu-58 to Gln-58 substitution. Successive additions of the leu-pro-ser triplet to the N-terminus restores Tp^R; the extension appears in some manner to stabilize the functional form of the protein. It may be that the extension allows proper folding, with consequent association of the monomer into the active tetramer. Whether any successive lengthening of the N-terminus could provide the same function is not known. The leu-pro-ser triplet exists naturally at position 18-20 of the R751 plasmid DHFR, but exists as leu-pro-leu in the R388 DHFR and as phe-pro-ser in the R67 protein (Flensburg and Steen, 1986; Stone and Smith, 1979; Swift et al., 1981). No repeat of any comparable triplet sequence is evident in the N-terminal regions of these natural enzymes.

Enhanced accumulation of proinsulin in *E. coli*, obtained by fusing a coding sequence for a short stretch of certain homo-oligopeptides to the 5′ end of the proinsulin gene, has previously been reported (Sung et al., 1986). In the Sung et al. (1986) study a homo-oligopeptide of serine increased the intracellular accumulation of proinsulin; however, the effect was highly dependent on the ser codons used. No enhancement was observed for homo-oligopeptides of either leucine or proline.

This study shows the importance of strain variations in the ability of plasmid-bearing strains to produce a mutant protein. The stresses of heat and high level protein production or abnormal protein production act to increase the proteolytic activity of the cell, especially through the *htp*R-*lon* system (Goff and Goldberg, 1985). These effects coupled with the stress of high plasmid copy number can act to adversely impact the production of labile proteins by increasing the degradation rate. The notion that we were adding to these stresses by the high level synthesis of a factor (a suppressor tRNA) which would independently enhance the production of abnormal proteins through its action on normal mRNAs may provide an explanation for the increased degradation found under these circumstances. This idea would be consistent with findings that conditions which favor the production of abnormal proteins lead to increased protease activity. This result could extend the number of stresses that lead to increased protease activity and is interesting in light of the recent connection proposed between ribosome function and heat shock sensing (VanBogelen and Neidhardt, 1990). The effect of excess suppression was not examined in the thorough study of other defective protein inducers of *lon* (Goff and Goldberg, 1985). There is also a possibility that the degradation of Gln-58 DHFR may not be acting through the usual heat response degradation enzymes. Certainly other candidates exist for the proteases that might be involved; the detailed nature, specificity and regulation of these are not well known. Perhaps specialized mutant protein systems with strong selections such as the trimethoprim resistant DHFR can be used to fruitfully investigate cell proteases.

This study also shows the ability of second site revertants to arise and stabilize a mutant, labile protein. This is an important idea when so many site directed mutants are made and studied. On the one hand these can compromise interpreta-

tion from experiments. Since some desired mutant proteins may well be labile, it is especially important to characterize the gene completely to notice if any other changes have accumulated which may act to modify the effect of the original amino acid change introduced. However, the second site revertants can be quite useful in a practical sense, since if they affect only the ability of the protein to be accumulated but do not affect the role of the original alteration, this type of stabilizing revertant can allow sufficient quantities of the protein to be isolated for detailed enzymatic studies.

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